Rhodopsin Chromophore Exchanges among Opsin Molecules in the Dark

Dennis M. Defoe* and Dean Bok

Turnover of rhodopsin chromophore in vertebrate visual cells has been explored by light microscope autoradiography (LMARG) and radiobiological techniques. Retinol-binding protein (RBP) was isolated from human serum, its native ligand removed and replaced with [3H]-retinol. After reconstitution, [3H]-retinol-RBP was reassociated with prealbumin (PA), and the protein complex injected intravenously into dark-adapted animals. After selected intervals in the dark, animals were killed, and ocular tissues dissected under infrared illumination. Eyecups from frogs and mice were fixed (4 C) and after in situ reduction of the chromophore-protein linkage of rhodopsin with borane dimethyl amine (BDMA), processed histologically to retain lipids, or alternatively to extract them with chloroform-methanol (C-M), and LMARG performed. Rhodopsin was purified from detergent-solubilized mouse retinas by Concanavalin A (Con A) affinity chromatography and analyzed for radioactivity. Autoradiographic labeling of frog rod outer segments (ROS) was first detectable at 1 day postinjection, increasing over the duration of the experiment. At all times, label was distributed throughout the organelle in a diffuse pattern, although in certain cases a band of silver grains was also evident at the proximal end of the ROS, the site of new membrane assembly. Similar autoradiographic patterns were noted in mouse rods, although the kinetics of labeling differed in certain respects. In biochemical experiments, incorporation of [3H]-retinol into mouse rhodopsin was seen to occur very rapidly (<30 min), without an appreciable lag period. We interpret the diffuse labeling of ROS to result from an exchange in the dark of [3H]-vitamin A aldehyde for unlabeled opsin-bound chromophore, whereas the formation of a reaction band no doubt reflects the continual renewal of ROS membrane occurring in the dark. With respect to the former, the turnover of chromophore qualitatively resembles that found for membrane fatty acids. Invest Ophthalmol Vis Sci 24:1211-1226, 1983

Vertebrate retinal photoreceptors (rods and cones) are highly specialized neural cells capable of the absorption of light and its transduction into an electrical signal. The photosensitive visual pigment rhodopsin, which is responsible for light trapping, exists as an integral membrane component of rod photoreceptor outer segment discs.1 A conjugated glycoprotein,2 it also binds covalently in its unbleached state one molecule of 11-cis-retinal, which serves as the chromophore. Upon the interaction of light with rhodopsin, visual pigment photolysis takes place with the protein and its chromophore physically separating. In order for visual sensitivity to be maintained, reformation of the complex, or regeneration, must take place.

Much current interest has focused upon the dynamic nature of rod outer segments since the discovery that outer segment disc membranes are continually assembled at their proximal end and shed from their distal tip.3'4 The relative molecular simplicity of ROS membranes coupled with the exquisite compartmentation of photoreceptors make it a particularly favorable system for studying membrane biogenesis.5 These features have been exploited in a series of autoradiographic and radiobiological experiments investigating the turnover of ROS membrane constituents.5-11 Although much information is available concerning the synthesis of visual pigment apoprotein, its conjugation with oligosaccharide residues and assembly into membrane, relatively little is known about the cellular events involved in the use of vitamin A by photoreceptors for regeneration and renewal.

Most of the vitamin A in plasma is present as all-transretinol bound noncovalently to a specific carrier protein called retinol binding protein (RBP).12,13 Under normal conditions, RBP, in turn, associates as a
protein complex with plasma prealbumin (PA).\textsuperscript{12,13} Uptake of vitamin A by the retina is known to occur through the specific interaction of holo-RBP with its plasma membrane receptor located on the basolateral (choroidal) surface of the retinal pigment epithelium (RPE).\textsuperscript{14–16} However, the details of its intraretinal handling and shuttling between RPE and photoreceptors are still incompletely understood.

We have used \([^{3}H]\)-retinol, delivered intravascularly as a complex with serum RBP and PA to explore the turnover of visual pigment chromophore autoradiographically and by radiobiochemical methods. Using a recently developed technique that stabilizes the retinal-opsin bond,\textsuperscript{17} we report on the pattern, location and kinetics of turnover and its influence by light. The implications for ROS turnover and the visual process are also discussed.

Materials and Methods

Preparation of \([^{3}H]\)-retinol-RBP-PA for Injection into Animals

Serum RBP and PA were purified from outdated human plasma (UCLA Hospitals Blood Bank) by a modification of the procedure of Peterson.\textsuperscript{13,18} Removal of native retinol from holo-RBP was accomplished by exhaustive extraction with absolute ethanol\textsuperscript{18} or, alternatively, by irradiation of solutions of RBP with monochromatic light of 330 nm using an Amino-Bowman spectrofluorometer to cause photodestruction of the ligand. The resulting apo-RBP was used for reconstitution with \([^{3}H]\)-retinol.

\([1,1^\text{12},1,2^\text{H}]\)-all-trans-retinoic acid (1.24 Ci/mmole) and \([1,1,2^\text{H}]\)-all-trans-retinoic acid (3.33 Ci/mmole) were generous gifts of Hoffman-LaRoche (Basle, Switzerland). Tritiated retinol was prepared from labeled retinoic acid by lithium aluminum hydride (LiAlH\textsubscript{4}) reduction of \([^{3}H]\)-methyl-retinoate.\textsuperscript{19} Reconstitution of \([^{3}H]\)-retinol-RBP and recomplexing of the holoprotein to PA was generally accomplished as previously described.\textsuperscript{18} In certain experiments, \([^{3}H]\)-retinol-RBP was purified on a column of PA-Sepharose 4B (1.5 \times 18 cm),\textsuperscript{20} prior to recomplexing.

Experimental Protocols

 Autoradiography: Frogs (Rana pipiens, 20–25 g ea) were dark adapted for 2 days prior to the experiment. Animals were anesthetized lightly by immersion in the dark in a 0.02% solution of m-aminobenzoic acid ethyl ester (MS-222, Calbiochem., San Diego, CA) in tap water. The head and upper abdomen of each frog was then hooded with a snugly-fitting opaque, black plastic sack to prevent light exposure during injection. Under a dissecting lamp, an abdominal musculocutaneous vein was exposed by skin incision and each frog injected intravenously with \([^{3}H]\)-retinol-RBP-PA solution (0.73 Ci/mmol, 0.5 \mu Ci/\mu l) at a calculated dose of 2.5 \mu Ci/g body wt. Afterwards, the skin wound was sutured and the animals immediately returned to the dark. Unless otherwise specified, all manipulations of animals were carried out in the dark or with the aid of a head-mounted infrared (IR) image converter (FJW Industries, Mt. Prospect, IL). Frogs injected in this way were maintained for up to 8 wks at 22 C in a completely light opaque black lucite container which, however, allowed free exchange of air and water through a baffle system. Periodically, the animals were force-fed meal worms.

At selected intervals (30 min, 6 hrs, 1 day, 1 wk, 2 wks, 4 wks, 8 wks) one animal was killed by decapitation and its eyes removed under IR light. Posterior eyecup preparations were obtained by making a circumferential cut around the ocular equator and removing the cornea, lens, and ciliary body (anterior segment), using a dissecting microscope fitted with dual IR image converting tubes. Following dissection, eyecups were placed immediately in ice-cold phosphate-buffered 4% formaldehyde (0.085 M sodium phosphate, pH 7.2, containing 3% sucrose) and immersion-fixed overnight in the dark at 4 C, with gentle agitation. For certain time points, a single additional dark-adapted frog was anesthetized, and one eye enucleated and dissected under infrared light and subsequently fixed in the dark. After recovery from anesthesia, the animal, with its other eye still intact, was light adapted for 1 hr at 40 ft cdls. The frog was then killed, and its remaining eye removed in the dark and treated exactly as outlined above.

Preparation and handling of adult male mice (C57Bl, 20–25 g ea) was essentially as described for frogs. Dark-adapted animals were placed in a hooded restrainer and, while fully conscious, were injected in a lateral tail vein with \([^{3}H]\)-retinol-RBP-PA in physiologic ionic strength buffer (2.42 Ci/mmol, 0.56 \mu Ci/\mu l) at a calculated dose of 1.6 \mu Ci/g body wt. Afterwards, animals were maintained at 22 C in the same dark box used for frogs.

At intervals (30 min, 2 hrs, 6 hrs, 1 day, 4 days, 10 days), one or more animals were killed by cervical dislocation and their eyes removed and dissected under IR light. Eyecups were fixed in the dark overnight by immersion in 1% formaldehyde, 1% glutaraldehyde in 0.1 M sodium phosphate, pH 7.2 (4 C), agitated.

For light-adaptation controls, one animal from selected time points was anesthetized by intraperitoneal injection of 0.09 mg/g body wt nembutal, and one eye removed under IR illumination. The other eye...
of the intact, fully conscious animal, after pupillary dilation with cyclogyl (cyclopentolate-HCl), was then light exposed for 1.5 hrs at 40 ft cdls. After enucleation and fixation (dark), eyecups from both light- and dark-adapted animals were processed for light microscope autoradiography as described below.

**Biochemistry:** Dark-adapted mice (C56Bl) were injected with [3H]-retinol-RBP-PA solution (2.42 Ci/mmole, 0.23 μCi/μl) at a dose of 0.6 μCi/g body wt. All procedures were performed under strict dark conditions exactly as previously outlined. After maintenance in the dark box, animals were killed in groups of four and their retinas removed in IR light. Retinas from each time point were solubilized overnight in the dark at 4 C in 0.5 ml of 0.1 M sodium acetate, pH 6.0, containing 0.1 M dodecyl trimethyl ammonium bromide (DTAB) (Eastman Organic Chemicals, Rochester, NY), 0.1 M NaCl and 1 mM each of CaCl2, MgCl2 and MnCl2. DTAB was recrystallized from acetone before use. After centrifugation at 27,000 X g, clear supernatants were drawn off and used for analysis of rhodopsin (see below).

**Processing of Tissues for Autoradiography**

Fixed eyecups were dissected under IR illumination into smaller segments that were then separated into two groups and processed in the following ways: (1) Most of the tissue was reduced in the dark at pH 1.5 with borane dimethyl amine (BDMA)17; and (2) the remaining eyecup material was kept in the dark at 4 C prior to postfixation and embedding. Both reduced and unreduced eyecup pieces were in turn subdivided into two groups and each processed separately in order to either retain or extract cellular lipids. The lipid preservation method was based upon the rapid dehydration procedure of Stein and Stein21 as modified by Bibb and Young.4 For lipid extraction, tissue pieces were exhaustively extracted with chloroform:methanol (C-M) (2:1, vol:vol) and embedded as described previously.6 All tissue processing fluids from fixation, BDMA-reduction, dehydration and embedding steps were saved for analysis by liquid scintillation counting.

Mouse eyecups were reacted with BDMA in the dark as previously described, and processed according to the lipid preservation protocol. Spurr embedding medium (Polysciences, Inc., Warrington, PA), however, was substituted for Araldite because of its lower viscosity and superior infiltration properties.

**Determination of Radioactivity in Histologic Tissue Processing Fluids**

Aliquots of each of the buffer solutions and reagent changes reserved from the processing of frog eyecup tissue for autoradiography were diluted in PCS II scintillation cocktail (Amersham Corp., Arlington Heights, IL) and counted by liquid scintillation spectrometry. An indirect estimate of the amount of tissue processed according to each protocol was made by measuring the retinal area in each case. Radioactivity removed during processing was then calculated as DPM/unit area of eyecup and expressed as a percentage of the total amount of extractable radioactivity. The latter was taken to be the normalized radioactivity measured when fluids from unreduced, lipid-extracted tissue were analyzed.

**Light Microscope Autoradiography**

Light microscope autoradiography was performed on 0.5 μm sections of eyecup tissue according to previously published methods.4 For those frog tissues processed according to the lipid extraction protocol, as well as a series of mouse eyecup control slides, the plastic embedment of sections was removed by incubation in 1% saturated NaOH in absolute ethanol prior to exposure and development. Plastic was not removed from material that was processed by the lipid preservation method. Exposure times were for 1–300 days (frog eyecup) and 1–3 weeks (mouse eyecup). Sections were stained with Giemsa (Polysciences, Inc., Warrington, PA) and examined in a Zeiss Photomicroscope III (Carl Zeiss, New York, NY).

For quantitative analysis, appropriate fields of retina were photographed and printed at a final magnification of 1800X. Values for mean silver grain densities (in grains/unit area) were then obtained after subtraction of background. Rod outer segments analyzed were selected randomly from twenty sections obtained from at least two separate eyes.

**Rhodopsin Purification and Analysis**

Isolation of rhodopsin from detergent-solubilized mouse retinal extracts was accomplished on 250 μl columns of Con A-Sepharose 4B (Pharmacia, Piscataway, NJ) by the method of van Breugel et al.22 All procedures took place at 4 C, either in darkness or under dim red light. Column fractions were measured for absorbance at 278 and 500 nm, and the concentration of rhodopsin present calculated using the value E500 = 40,500 M⁻¹ cm⁻¹. Aliquots of each fraction were then counted by liquid scintillation spectrometry. Specific activity determinations for peak rhodopsin fractions were calculated as radioactivity (in DPM) divided by the total amount of visual pigment (in nmoles).
Fig. 1. Light microscope autoradiograms of eyecup sections from dark-adapted frogs injected with [3H]-retinol-RBP-PA. Tissues were fixed in the dark and reduced with BDMA prior to osmication and embedding according to the lipid preservation protocol (see materials and methods). Exposure time 3 days (bar, 10 μm, X790). a, 30 min after injection. No labeling of photoreceptor or RPE structures above background is seen. (pe, pigment epithelial cell; os, outer segment of rod; is, inner segment of rod; cb, cell body of photoreceptor). b, 1 day after injection. Pigment epithelium oil droplets (od) are significantly radioactive. A few scattered silver grains are present over rod outer segments. c, 2 weeks after injection. Rod outer segments are heavily labeled in a diffuse pattern. RPE oil droplets remain radioactive; however, reaction product is sparse over receptor inner segments and cell bodies. d, 8 weeks after injection. ROS continue to accumulate [3H]-retinol with no evidence for localized incorporation. A phagosome (ph) is shown which is labeled to a similar extent as adjacent outer segments. Particularly noticeable in this micrograph is the greater length of outer segments, a phenomenon that is not uncommon in retinas dark-adapted for prolonged periods.

SDS-Polyacrylamide Gel Electrophoresis

Portions of crude retinal extract, pooled column washes, and purified rhodopsin fractions from Con A-Sepharose 4B chromatography were diluted in 0.1 M sodium acetate buffer (containing 0.1 M DTAB and 0.1 M NaCl), acidified to pH 1.5. All solutions were incubated in the dark at 0 C for 5 min. Afterwards, solid BDMA was added to each at a final concentration of 20 mg/ml. Following incubation for 2 hrs at 4 C, the reduced solutions were further diluted in buffer adjusted to pH 6.0 and brought into the light. Prior to SDS gel electrophoresis, DTAB was removed from samples by exhaustive dialysis against 5 mM Hepes buffer, pH 6.6. The detergent-free material was then collected by centrifugation (34,800 x g) and the resulting pellets solubilized in 2.5% SDS and 2.5% β-mercaptoethanol. Discontinuous gel electrophoresis was carried out using 10% polyacrylamide slabs. After staining with Coomassie Bril-
lant Blue, individual lanes were scanned at 560 nm using a Beckman ACTA MVI spectrophotometer equipped with a gel scanning apparatus. Slices of stained gel were digested in 30% hydrogen peroxide for 3 hrs at 60°C,25 diluted in PCS II cocktail and counted.

**Results**

**Pattern and Kinetics of [3H]-Retinol Incorporation into Frog Retinas**

Eyecups reduced with BDMA and subsequently processed to retain as much lipid-soluble material as possible, were used to evaluate the overall incorporation of [3H]-retinol following its intravenous injection. The series of light microscope autoradiograms shown in Figure 1 represents the labeling patterns obtained for tissues of dark-adapted animals at several selected time points. Because the identification of [3H]-retinol incorporation specifically into visual pigment was of primary interest in this study, autoradiography was also performed on identical retinal tissues which had been reduced to fix rhodopsin chromophores in place but which were subsequently extracted with C-M to remove free vitamin A compounds such as retinyl esters and retinyl phospholipids. Quantitation of this data was performed by grain density analysis and appears in Figure 2.

The earliest interval sampled was 30 min after injection of [3H]-retinol-RBP-PA. At this time, (Fig. 1a), as well as at 6 hrs, little if any radioactivity was detectable anywhere within the retina, resulting in an initial lag period of incorporation (Fig. 2). By 1 day, however, a significant number of silver grains was observed over RPE oil droplets, although labeling elsewhere within the epithelial cells was absent. The 1-day autoradiogram also showed the first indication of specific reaction over rod outer segments (Fig. 1b). No evidence for suborganellar localization of radioactivity was discernible at this time, however. Instead, a diffuse sort of labeling was present.

From 1 day to 1 week, grain densities over red ROS rose steadily, increasing approximately 20-fold. This increase occurred during a time when accumulation of [3H]-retinol within RPE oil droplets was also taking place, and blood radioactivity had declined to less than 5% of its initial value (data not shown). Thereafter, ROS incorporation was approximately linear up to 2 weeks. Retinas from 1- and 2-week animals showed that incorporation of [3H]-retinol into the outer segments of rod photoreceptors had occurred in an apparently random manner (Figs. 1c, 3a). This was true of both red rods and the less numerous green rods. The latter possess a distinct rhodopsin with peak absorbance shifted to shorter wavelengths26 and are distinguishable morphologically from red rods by their shorter, more sclerally displaced outer segments. In spite of the high degree of outer segment labeling, there was little evidence for significant amounts of radioactivity appearing within the inner segments of photoreceptors. Therefore, it seems unlikely that a precursor-product relationship exists which involves these two cell compartments.

By 4 weeks postinjection, grain densities leveled off and remained roughly the same through 8 weeks. In autoradiograms of the latter time point (Fig. 1d), the pattern of labeling was much the same as seen previously. Grain counts performed on green ROS revealed an overall similarity in their labeling kinetics to that found for red ROS, described approximately as a sigmoidal curve (Fig. 2). However, green rods accumulated label faster initially, so that by 1 day grain densities were more than 3X those of red rods (0.039 ± 0.001 vs. 0.012 ± 0.001 grains/unit area).

**Effect of Light Adaptation and Lipid Extraction on the Pattern of [3H]-Retinol Labeling**

If the bulk of silver grains present over ROS in autoradiograms represents radioactive rhodopsin molecules, then the labeling pattern observed should be altered by light because of rhodopsin bleaching. Accordingly, eyecup tissues of a 1-week animal, which were reduced with BDMA and either lipid-preserved or alternatively, lipid-extracted were prepared and LMARG performed. However, while one of the eyes was taken out and processed in the dark, the other eye of the same frog was light-exposed prior to processing for autoradiography. From inspection of
Fig. 3a & b. Comparison of light microscope autoradiograms from dark- and light-adapted frog eyes. Specimens, obtained from an animal killed 1 week after injection, were BDMA-reduced and processed to retain lipids. Exposure time 3 days (bar, 10 μm, X790). a, Dark-adapted retina. ROS are substantially labeled as are RPE oil droplets (od). A green rod (gr) is also evident. b, Light-adapted retina. Silver grain densities over outer segments are somewhat reduced compared with those in a, while oil droplets (od) remain heavily labeled. c & d, Comparison of light microscope autoradiograms from reduced and unreduced dark-adapted frog eyes after extraction with C-M. Tissue was obtained from a 1 week animal. Exposure time 3 days (bar, 10 μm, X790). c, BDMA-reduced eyecup. Although the tissue is essentially depleted of lipid, ROS are substantially labeled (compare with a). Grain densities over photoreceptor inner segments and cell bodies do not exceed background levels. RPE oil droplets are removed along with their label. d, Unreduced eyecup. Rod outer segments are unlabeled, as are all other retinal structures.

In the autoradiograms of lipid-preserved tissue, a subtle change in the distribution of silver grains within the RPE photoreceptor unit was seen (Figs. 3a, b). The light-adapted retina displayed a small decrease in labeling of rod outer segments. Although not quantified, the decline was less than might be expected given the fact that approximately 50% of the visual pigment molecules would be bleached under the conditions employed. Vitamin A is known to cycle continuously between retina and pigment epithelium during light adaptation. Therefore, it is possible that a large decline in ROS radioactivity is not observed because the exchangeable pool of vitamin in the epithelium is of a very high specific activity. Differences in labeling of ROS from light- and dark-adapted eyes were also seen in BDMA-reduced, lipid-depleted tissue (data not shown), as might be expected if the light-sensitive changes are a reflection of retinal bound to opsin.

Concomitant with the alterations in outer segment labeling were changes in the structure of RPE oil droplets. These organelles were larger and more...
prominent in light-adapted retinas, but remained substantially radioactive. The lack of a significant change in silver grain patterns over either photoreceptor inner segments, RPE cytoplasm or any other retinal structure is consistent with the idea that all-trans chromophore liberated by bleaching was taken up by epithelial cells and stored in oil droplets. This conclusion is in agreement with evidence obtained by others using different methods.27-29

The effect of lipid extraction on the autoradiographic pattern of BDMA-reduced retinas is seen in Figure 3c. The C-M treatment is responsible for the poor morphologic preservation of retinas in general and ROS in particular. Nevertheless, in most cases, structural features are readily identifiable. Even though nearly 93% of the outer segment phospholipids are removed by this procedure,30 the bulk of the radioactivity present in lipid-containing specimens appears to be retained. Unreduced retinas, on the other hand, do not retain their label (Fig. 3d). This would seem to indicate that tritiated vitamin is covalently bound to protein and, therefore, most likely exists as reduced rhodopsin (N-retinyl-opsin). These autoradiograms in addition reveal that large oil droplets and their radioactive contents were completely removed by organic solvents, and that labeling of RPE cytoplasm has decreased.

[3H]-Retinol Incorporation into ROS-Renewed vs. Non-renewed Membrane

ROS radioactivity, from LMARG, generally appeared to be distributed randomly throughout the outer segment. However, in overexposed autoradiograms a subtle change in the pattern of incorporation was manifest at 1 week, where a small but definite enhancement of labeling was recognizable over the basal portion of red rod outer segments (Fig. 4). The proportion of organelle area exhibiting the increased grain density was roughly 10%, approximately the amount of disc membrane that would be added during the postinjection period. Assuming that frog photoreceptors renew their ROS completely in about 8-10 weeks,3 labeled discs formed at 1 week would be expected to migrate approximately one-fourth to one-half the way along the outer segment at 2 and 4 weeks, respectively.

In order to determine the proportion of ROS radioactivity that was incorporated into newly synthesized visual pigment, and to determine if band migration was occurring, quantitative analysis of selected light microscope autoradiograms was performed. Grain densities were measured independently over portions of proximal outer segment membrane which were calculated to have been renewed during the period of the experiment, and compared with similar measurements for the remainder of the ROS. At 1 week, the basal 10% of disc membrane was specifically labeled, on the average, 1.4 x that in other parts of the organelle (Table 1). The other time point sampled, 2 weeks, did not reveal significant differences, however. This fact suggests that some sort of redistribution of radioactivity occurred during the period subsequent to band formation.

Assessment of Tissue Processing Protocols

Much of the autoradiography of frog eyecup was performed on tissues that were reduced with BDMA and processed according to a modified dehydration and embedding protocol designed to retain lipids. Although in micrographs most of the radioactivity seemed to be preserved in situ, quantitative verification for this conclusion was also sought. Accordingly, the fate of labeled vitamin A present in tissues was followed throughout histological processing. All fixation, dehydration and infiltration fluids from separately treated eyecup pieces were collected and measurements of radioactivity taken and normalized as described in the materials and methods.

Results of this analysis are shown in Figure 5. When reduced eyecups were processed to retain maximal amounts of lipid soluble compounds, only 10% of the radioactivity originally present was recovered in fluid washes. However, when eyecups were reduced
Table 1. Quantitative light microscope autoradiography: Incorporation of $^{3}$H-retinal into proximal versus distal frog rod outer segment discs

<table>
<thead>
<tr>
<th>Time point</th>
<th>Newly-synthesized membrane</th>
<th>Pre-existing membrane</th>
<th>$P$ level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ROS grain density (grains/unit area)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$^{3}$H-retinal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(grains/unit area)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>0.979 ± 0.079 (basal 10%)</td>
<td>0.653 ± 0.034 (remaining 90%)</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>2 weeks</td>
<td>1.704 ± 0.103 (1st 10%)</td>
<td>1.725 ± 0.111 (2nd 10%)</td>
<td>&gt;0.90</td>
</tr>
<tr>
<td></td>
<td>1.681 ± 0.050 (remaining 80%)</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Autoradiograms were prepared from eyecups of dark-adapted animals that were reduced with BDMA and extracted with C-M. Grain counts were separately performed on regions of red ROS estimated to have been assembled during the postinjection period and on regions assembled prior to injection. The newly synthesized membrane was analyzed in increments of 10% of the total area of the ROS. Therefore, the pre-existing membrane in each case represents [100 − (the number of increments × 10)%] of the ROS area. Values indicated are the mean (±SEM) (n = 48). $P$ levels result from two-way analysis of variance of the means of each time point.

with BDMA, but subsequently lipid-depleted, about one-third of the tissue radioactivity was extractable. Since, in autoradiograms most of the ROS label was still retained, the counts removed must derive primarily from pigment epithelial cells and be due to free vitamin A compounds or those bound noncovalently to protein. Autoradiograms from eyecups that were unreduced but lipid-extracted showed no evidence of specific labeling. Therefore, the assumption that nearly complete extraction of nonopsin radioactivity took place in reduced specimens is probably valid.

**Vitamin A Metabolism by Mouse Retinas: Autoradiographic Results**

Because of several advantages they offer, including a faster rate of disc synthesis and smaller intraretinal pools of vitamin A, mice were also injected with $^{3}$H-retinol-RBP-PA and their retinas analyzed by LMARG. The set of autoradiograms shown in Figure 6 is a study of the changing pattern of silver grain distribution with time within the photoreceptor-RPE unit. Dark-adapted animals were used throughout and eyecup tissues have been BDMA-reduced and processed to retain lipids.

At the earliest time intervals, 30 min (Fig. 6a) and 2 hrs, a small amount of labeling above background levels was evident within visual cell inner and outer segments. It was not until 6 hrs, however, that significant incorporation into ROS was apparent (Fig. 6b). As in autoradiograms of frog tissue, labeling was diffuse, and present throughout the organelle. There was little evidence at these times for detectable radioactivity in pigment epithelial cells. Autoradiograms of eyecups from animals killed at 1 day post-injection (Fig. 7c) revealed a further increase in ROS incorporation. Although the distribution of reaction product was very similar to that at 6 hrs, it was possible to detect in certain rods the presence of clustered silver grains over the basal regions of outer segments. The pattern of reaction was much more striking at 4 days (Fig. 6c). Clearly, a greater incorporation of $^{3}$H-retinol has occurred throughout the ROS, but this was especially true of the basal one-third of disc membranes. This result indicates that a great deal of tritiated vitamin was being utilized for new visual pigment synthesis. At 10 days (Fig. 6d), the apical one-third of the outer segment was heavily labeled, indicating that the reaction band previously formed had been displaced due to membrane renewal. Unlike frog retinas, in which two distinct classes of rod photoreceptors are found, mouse retinas possess only one type of rod.

In control experiments, similar to those in frogs, the bulk of autoradiographic label could be shown to correlate with N-retinyl-opsin. For example, in light-
exposed eyes (Fig. 7b), the concentration of silver grains over rod outer segments was diminished significantly relative to that of eyes kept in the dark (Fig. 7a). In addition, all indications of a discernible reaction band had disappeared. On the other hand, RPE cells from the same light-adapted eye were now substantially labeled. This fact strongly suggests that the loss of ROS radioactivity is reflected in uptake by the adjacent epithelium. This is what would be expected if the bleaching of radioactive rhodopsin molecules were taking place.28

Difficulties in maintaining a stable interaction between neural retina and pigmented layers during fixation in formaldehyde alone necessitated the additional inclusion of glutaraldehyde in these experiments. However, the fact that the latter is a strong cross-linking agent precluded the use of C-M as an effective extractant of amino lipids. In an attempt to overcome this restriction, some embedded and sectioned specimens were treated with alcoholic NaOH, a powerful saponifying reagent, before autoradiography. This procedure, which is designed to deplasticize the tissue, might also be expected to remove a certain amount of lipid-soluble material.
Fig. 7a & b. Effect of dark- and light-adaptation on the autoradiographic pattern of mouse retinas. An animal injected with \([\text{H}]-\text{retinol}\)-RBP-PA and maintained in the dark was killed at 4 days. One eye was enucleated in the dark while the contralateral eye was light-exposed. Both eyes were fixed, BDMA-reduced and processed to retain lipids. Exposure time 14 days (bar, 10 \(\mu\)m, X1050). a, Dark-adapted retina. Radioactivity is concentrated over basal ROS discs in a reaction band while the RPE is unreactive. b, Light-adapted retina. Labeling of outer segments is substantially reduced and distributed uniformly. Grain densities over regions of the RPE have increased compared with dark-adapted control. c & d, Effect of removal of plastic embedment on the autoradiographic pattern of mouse retinas. Eyecup from an injected animal killed after 1 day was processed as described in Figure 6. Exposure time 14 days (bar, 10 \(\mu\)m, X1050). c, Undeplasticized tissue section. Labeling of outer segments is pronounced, while uptake of radioactivity into inner segments and cell bodies is moderate. d, Deplasticized tissue section. Although grain densities over ROS are diminished relative to controls, much radioactivity is retained. Moreover, the distribution of this outer segment label matches that for undeplasticized retina. Labeling of photoreceptor inner segments and cell bodies appears to be largely removed.

As shown in Figure 7d, practically all of the radioactivity present in the inner segments and cell bodies of photoreceptors in undeplasticized specimens (Fig. 7c) had disappeared following treatment. There was comparatively little difference, however, in the labeling pattern of ROS, although at earlier time intervals, a noticeable decline in grain density throughout the organelle was evident (data not shown). This latter point indicates that some of the radioactivity originally taken up must not have been covalently bound to opsin. Presumably this represents material that is a part of the small pool of vitamin A compounds known to exist in outer segments.31

Quantitative analysis of deplasticized tissue section autoradiograms was carried out to determine if, at early time intervals, preferential sites of \([\text{H}]-\text{retinol}\) incorporation into ROS existed. At 30 min, 2 hrs and 6 hrs the number of silver grains in the apical, middle,
and basal one-third of outer segments was approximately equal. It was only by 1 day, when a significant amount of newly synthesized discs had been formed, that a large proportion (50%) of the radioactivity was detected in basal ROS regions.

Vitamin A Metabolism by Mouse Retinas: Incorporation of [3H]-Retinol into Rhodopsin

In conjunction with autoradiographic experiments, radiobiochemical studies were also undertaken to directly measure the specific incorporation of [3H]-retinol into visual pigment. Mice were injected and maintained in the dark exactly as described for the previous experiments. Their retinas were then isolated and, after extraction into detergent solution, rhodopsin was purified by affinity chromatography.

![Figure 8](image1.png)

**Fig. 8.** Purification of rhodopsin from a mouse retinal extract by chromatography on Con A-Sepharose 4B (see materials and methods for details). The recovery of specific absorbance was better than 90%. The measurement of A280 for the initial effluent fractions possessed a considerable light scattering component and thus does not strictly reflect protein content. Also, a large proportion of the radioactivity in these fractions derives from isotope trapped in retinal blood spaces and does not represent intraretinal pools of vitamin A. Data shown are for the 6-hr time point that was typical of other intervals.

![Figure 9](image2.png)

**Fig. 9.** Analysis by SDS-polyacrylamide gel electrophoresis of mouse retinal extract after fractionation by affinity chromatography. Only a single major polypeptide (M, 35,000) is evident (lower gel lane). Minor bands of M, 70,000 and 150,000 are oligomers of opsin. Molecular weight standards indicated in the upper gel lane are serum albumin, SA (68K); ovalbumin, OV (43K) and chymotrypsinogen, CH (25.7K). Peak fractions from Con A-Sepharose affinity columns were also reduced with BDMA before solubilization in SDS and gel electrophoresis. Upon slicing of the gel and analysis for radioactivity, only the 35,000 molecular weight species (N-retinyl-opsin) and its oligomers are radioactive (graph).

The results of elution following Con A-Sepharose chromatography (Fig. 8) were similar to those previously described for crude ROS extracts.\(^2\) Ratios of A280/A500 were in the range 2.4–2.6, and recovery of rhodopsin based on A500 was usually greater than 90%. The SDS-gel pattern of isolated pigment (Fig. 9) indicated that the protein eluting with \(\alpha\)-D-methylmannoside was indeed greatly purified. A major protein-stainable band was evident, whose apparent molecular weight (35,000) is close to the accepted value for opsin. In addition, opsin dimer (Mr 70,000), an
artifact commonly seen in preparations of this type, is also conspicuous.

Confirmation that the incorporation of radioactivity was in fact into rhodopsin was provided by analysis of SDS-gels in which the protein present had been reduced with BDMA. This is shown in Figure 9 for the rhodopsin peak fraction, where practically all of the radioactivity comigrated with the 35,000 molecular weight species (similar results were also obtained for gels of crude retinal extract). The minor radioactivity peak at 15 mm is due to the opsin dimer.

For analysis of the kinetics of \( ^3H \)-retinol incorporation, measurements of absorbance at 500 nm and radioactivity were performed on rhodopsin fractions from the columns of all time points examined. Visual pigment specific activities were calculated and expressed as DPM/nanomole, and the resulting values were plotted versus time in graphical form. As can be seen in Figure 10, specific incorporation was detectable at 30 min. The rate of labeling was relatively slow during this initial period, but thereafter increased rapidly. Approximately linear incorporation was noted from 30 min to about 1 day, at which time approximately 1% of the rhodopsin molecules in the retina were radioactive. Thereafter, the labeling rate began to decline, reaching a plateau by about 4 days.

Absolute values for the exchange of tritiated vitamin A with visual pigment chromophore can be estimated when several assumptions are made. First, for purposes of simplification, only two main compartments are considered: (1) the bloodstream into which labeled vitamin A molecules are introduced; and (2) the rod outer segments, whose opsin molecules are viewed as having direct access to circulating isotope. Since mouse retinas probably possess only very small pools of vitamin A in their pigment epithelium, the latter assumption is justified. Based upon an estimate for the total amount of retinol in the plasma of a 25 g mouse and the amount of radioactivity injected, the specific activity of the vitamin A pool in the blood at zero time can be calculated. Similarly, the total number of radioactive rhodopsin molecules in both retinas of such an animal can be found, and with the data that results, the following formula set up:

\[
A \text{ (the number of tritiated vitamin A molecules exchanged/unit time/retina)} = \frac{B \text{ (radioactivity in rhodopsin pool in } \mu\text{Ci})}{C \text{ (specific activity of blood vitamin A available for exchange)}}
\]

Because of the nature of the rhodopsin purification procedure used, it was not possible to distinguish between turnover due to chroomophore exchange and that due to de novo pigment synthesis. Nevertheless, by determining the rate of labeling at a very early interval (2 hrs) an estimate of incorporation due primarily to exchange was obtained. This value \((2.9 \times 10^9 \text{ molecules exchanged/minute/retina or about } 2 \times 10^3 \text{ molecules exchanged/minute/rod)}\) reflects a very active turnover of chromophore existing in the dark.

**Discussion**

**Use of \( ^3H \)-Retinol by Frog and Mouse Retinas**

A major goal of this study was to define unequivocally the sites of incorporation of \( ^3H \)-retinol into the visual pigment of photoreceptor cells. This could only be accomplished using a technique such as light microscope autoradiography in which retinas could be examined histologically. However, due to the lability of the retinal-opsin bond, special measures first had to be taken to insure immobilization of the chromophore in situ prior to tissue processing. This was accomplished by treating retinas with the reducing agent BDMA to convert N-retinylidene-opsin to its more stable form, N-retinyl-opsin. In addition, because turnover of visual pigment chromophoric groups takes place continually throughout the process of light adaptation, photobleaching and its effects had to be rigorously excluded. By performing all experimental manipulations of animals and tissues either in the dark or under infrared illumination, this latter requirement was also met.

When reduced, lipid-depleted retinas were inspected autoradiographically, labeling of visual cells

† See appendix for calculations.
was restricted to ROS. Similar results were obtained when mouse retinal sections were treated with alcoholic NaOH, a procedure that might be expected to remove noncovalently bound ligand. There was no evidence for incorporation of radioactive vitamin A into photoreceptor inner segments, and therefore it seems likely that rhodopsin is localized exclusively to the outer segments of these cells, as initially proposed by Hall and Bok. This fact raises an important point regarding the biosynthesis of visual pigment molecules. The opsin polypeptide synthesized in the inner segment must acquire its chromophore only after being first assembled into ROS disc membrane. In addition, since outer segment labeling was not evident until substantial amounts of radioactivity had accumulated within the RPE, labeled rhodopsin chromophores derive ultimately from vitamin A in the epithelium. This is consistent with previous findings that a specific plasma membrane receptor for holo-RBP exists on the choroidal surface of epithelial cells.

Somewhat different patterns of photoreceptor labeling were obtained when comparable eyecup tissues were processed in order to preserve lipid-soluble material. In such cases, specific silver grain reaction was also present in small amounts over inner segments and cell bodies. Although the identity of this label is unknown, potential candidates include ligand bound to those putative retinol- and retinal-binding proteins which have tentatively been localized to photoreceptors.

The kinetic labeling curves constructed from counts of frog green and red ROS reveal some interesting differences between these visual cells in the way they metabolize retinol. Although an appreciable lag period for initial incorporation was also noted, turnover of green rod visual pigment chromatophore occurred much more rapidly in the dark. Interestingly, Bibb and Young have obtained evidence that turnover of membrane phospholipids is also greater in green than in red rod outer segments.

One would certainly expect cone visual pigment also to incorporate triitated retinol; however, this could not be analyzed by light microscope autoradiography due to the very small size of frog cone outer segments.

That the label visualized in autoradiograms was primarily attributable to radioactive rhodopsin molecules is indicated by the following results. First, when the eyes of animals that were previously injected and kept in the dark were light adapted prior to killing and LMARG, grain densities over outer segments were reduced. Also, in parallel control eye cup tissue that was reacted with BDMA but subsequently extracted with organic solvents either before (frog) or after (mouse) embedding to remove lipids, ROS retained most of their radioactivity.

The dramatic difference between frog and mouse labeling patterns noted after relatively long time intervals requires some explanation. We believe the differential distribution of basal and apical label in the two species can be rationalized as follows. The frog possesses an enormous pool of vitamin A in the RPE oil droplets and an approximately 1- to 2-day renewal supply in the ROS. When a pulse of labeled retinol is injected, most of it enters these pools and thus is available for incorporation into rhodopsin for weeks. Mice, on the other hand, have no detectable stores of vitamin A. Thus, when a dose of [3H]-retinol is administered, it is both combined with newly synthesized opsin and exchanged for pre-existing pigment only during the period in which isotope is circulating in the blood. Thereafter, the only significant labeling must come about by exchange of chromophore within the retina. Also, since animals are maintained in darkness, the rate of disc assembly in frogs is reduced to 88% of that occurring in animals on a light-dark cycle whereas the rate in mice is unaffected. The net result is prolonged replacement of chromophore by exchange accompanied by slow disc assembly in the frog, and preferential entry of vitamin A label into newly synthesized discs accompanied by a relatively short period of exchange in the mouse. The reaction band is weak and soon randomized in the frog whereas a comparable degree of randomization of label never can develop in the mouse and so the band remains prominent.

**Chromophore Exchange**

In these experiments, evidence has been obtained for incorporation of exogenous vitamin A into rod outer segment rhodopsin when experimental animals are kept in total darkness. Although in certain cases labeling must be due to de novo synthesis of visual pigment, from the reaction patterns obtained in autoradiographic experiments, much of the label is associated with membrane not undergoing renewal. Furthermore, the biochemical results demonstrate specific incorporation at times temporally resolved from new disc synthesis. The most likely explanation for these results is that rhodopsin chromophores are continually turning over, exchanging with other opsin prosthetic groups and existing in equilibrium with retinal pools of vitamin A. This is in distinct contrast to visual pigment apoprotein which, once inserted into outer segment membranes, is not replaced.

Results qualitatively similar to those reported here have been obtained in autoradiographic experiments tracing the incorporation of radioactive phospholipid precursors. Upon delivering [3H]-glycerol and labeled
fatty acids such as [3H]-palmitate and [3H]-stearate intravascularly to frogs, Bibb and Young\(^6,7\) found that, as in the present study, labeled molecules appeared diffusely distributed throughout rod outer segments. These authors concluded that, in addition to de novo synthesis of bulk membrane, the ROS membrane is capable of both the interstitial replacement of whole phospholipid molecules, an apparently ubiquitous phenomenon,\(^37\) and the exchange of individual component fatty acids. It is generally believed, although not directly demonstrated, that such replacement may be necessary in order to remove damaged molecules that would otherwise function improperly. Unsaturated fatty acids, present in high amounts in ROS phospholipids\(^38\) are particularly prone to peroxidative damage due to reaction with oxygen radicals. In this regard, it is interesting that Dudley and Anderson\(^39\) have recently reported the presence in retinal fractions of a protein catalyzing the exchange of phosphatidyl choline between artificial unilamellar phospholipid bilayers and bovine rod outer segments. It is conceivable therefore, that the 11-cis-retinal molecule with its high degree of unsaturation is also susceptible to oxidative attack, which in turn may render it unable to react with opsin. Visual cells may, for example, possess an active mechanism for replacing 11-cis-retinal analogous to the exchange of phospholipids by phospholipid-exchange proteins in other tissues.\(^37\)

Rhodopsin, whether in isolated outer segments or in selected detergent extracts, has generally been reported to be stable at low to moderate temperature, as judged by the insignificant loss in visible absorbance over prolonged periods.\(^40\) Nevertheless, at elevated temperatures the pigment molecule undergoes a small but measurable bleaching in the dark.\(^41\) It is possible that a small degree of thermal isomerization may occur in vivo, converting 11-cis-retinal to the all-trans form and causing hydrolysis of the aldimine bond. The opsin that results would then be free to combine with fresh 11-cis isomer, regenerating rhodopsin. Bridges\(^31\) has recently detected a small pool of 11-cis-vitamin A in frog rod outer segments, which he suggests may serve as a limited source of readily available prosthetic groups.

If the exchange seen in the dark is in fact due to a steady thermal bleaching of rhodopsin molecules, this would have important consequences for visual transduction and the threshold perception of light. Since the only direct action of light on a visual pigment is isomerization of the chromophore, such thermal events may mimic the light response, producing false excitation events. In recent years, several authors have found evidence for spontaneous fluctuations in membrane voltage originating in photoreceptor cells in the dark.\(^42-45\) The conductance changes that underlie this so-called dark noise are believed to be due to the opening and closing of single ionic channels. Baylor and his colleagues\(^46-48\) have tentatively localized the source of the dark noise in rods to the outer segments themselves, using a suction electrode to draw up and electrically isolate single frog rod outer segments. From analysis of its temperature-dependence, they concluded that the noise, which has an average frequency of one event per 50 sec at 20°C, may ultimately be a result of the spontaneous bleaching of rhodopsin due to thermal isomerization. Calculations of the dark exchange of chromophore in mice in the present experiments indicate a much faster turnover of rhodopsin prothetic groups which cannot be accounted for by the different body temperatures of the two animals (see appendix). This high rate of dark incorporation of [3H]-retinol into visual pigment suggests that the photoreceptor cell may possess a mechanism for chromophore turnover that does not activate the transduction mechanism and lead to excitation.

**Acknowledgments**

The authors are most grateful for the technical assistance of Ms. Caryl Schecter and Ms. Marcia Lloyd. We also wish to thank Dr. Michael Kutner for help with the statistical analysis and Joanna Corley and Margaret Cantie for typing the manuscript.

**References**

9. Hall MO, Bok D, and Buchachar ADE: Biosynthesis and as...


APPENDIX

Calculation of the exchange rate of tritiated vitamin A and visual pigment chromophore in mouse retinas (analysis for the 2-hr time point):

Rhodopsin specific activity = 2.01 × 10⁻² µCi/nmole. Amount of rhodopsin/2 retinas (ave.) = 0.90 nmole
thus, amount of radioactivity/2 retinas as rhodopsin (B) = $2.01 \times 10^{-3} \mu\text{Ci/nmole} \times 0.90 \text{ nmole} = 1.82 \times 10^{-3} \mu\text{Ci}$

also, amount of $[^3\text{H}]$-retinol injected/animal (ave.) = 13.8 $\mu\text{Ci}$ (5.79 nmole). Amount of native (unlabeled) retinol in blood/animal (ave.) = 1.90 nmole/ml $\times$ 1.6 ml blood/animal = 3.05 nmole

thus, specific activity of blood vitamin A available for exchange (C) = $13.8 \mu\text{Ci} \div (5.9 + 3.05 \text{ nmole}) = 1.56 \mu\text{Ci/nmole}$

therefore,

$$A = \frac{B (1.82 \times 10^{-3} \mu\text{Ci})}{C (1.56 \mu\text{Ci/nmole})} = 1.17 \times 10^{-3} \text{ nmole exchanged/2 hrs./2 retinas}$$

or,

$$A = \frac{(1.17 \times 10^{-3}) \times (6.02 \times 10^{14} \text{ molecules/nmole})}{120 \text{ min} \times 2 \text{ retinas}} = 2.94 \times 10^9 \text{ molecules exchanged/minute retina}$$

and,

$$A = \frac{(2.94 \times 10^9) \times (1.63 \times 10^8 \text{ molecules of rhodopsin/rod})}{(2.71 \times 10^{14} \text{ molecules of rhodopsin/retina})} = 1.77 \times 10^3 \text{ molecules exchanged/minute rod}$$

Using the experimentally determined $Q_{10}$ of 3.46 for thermal noise in toad rod outer segments (Baylor et al, 1980), the theoretical rate of vitamin A exchange at 22 C can be calculated,

$$A_{22^\circ\text{C}} = (1.7 \times 10^3 \text{ molecules exchanged min rod}) \div (5.19) = 3.41 \times 10^2 \text{ molecules exchanged min rod}$$